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A new look at the hemolytic effect of local anesthetics, considering their real membrane/water partitioning at pH 7.4

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Abstract

The interaction of local anesthetics (LA) with biological and phospholipid bilayers was investigated regarding the contribution of their structure and physicochemical properties to membrane partition and to erythrocyte solubilization. We measured the partition into phospholipid vesicles—at pH 5.0 and 10.5—and the biphasic hemolytic effect on rat erythrocytes of: benzocaine, chloroprocaine, procaine, tetracaine, bupivacaine, mepivacaine, lidocaine, prilocaine, and dibucaine. At pH 7.4, the binding of uncharged and charged LA to the membranes was considered, since it results in an ionization constant (pK_{app}) different from that observed for the anesthetic in the aqueous phase (pK_w). Even though it occurred at a pH at which there is a predominance of the charged species, hemolysis was greatly influenced by the uncharged species, revealing that the disrupting effect of LA on these membranes is mainly a consequence of hydrophobic interactions. The correlation between the hemolytic activity and the LA potency shows that hemolytic experiments could be used for the prediction of activity in the development of new LA molecules.

Keywords: Local anesthetics; Erythrocytes; Hemolysis; Ionization; Partition; Membrane

1. Introduction

Local anesthesia is achieved by binding of anesthetic molecules to the voltage-gated sodium channels in the axon. Local anesthetics (LA) binding to the sodium channel inhibit the propagation of the action potential responsible for the nervous impulse [1–4]. Most LA are relatively hydrophobic ionizable amines that undergo partitioning into lipids [5]. Effects of LA on the structural and dynamic proper-

Abbreviations: BVC, bupivacaine; BZC, benzocaine; CLP, chloroprocaine; DBC, dibucaine; EPC, egg phosphatidylcholine; LA, local anesthetic; LA+, charged species; LA:, uncharged species; LDC, lidocaine; MVC, mepivacaine; *P*, partition coefficient; pK_w, ionization constant measured in water; pK_{app}, ionization constant, measured in water but in the presence of a membrane; PLC, prilocaine; PRC, procaine; *S*, solubility; TTC, tetracaine.

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ties of the membrane lipid region could be responsible for some of the toxic effects caused by these molecules [6,7].

We studied the interaction between ester (benzocaine), amino ester (chloroprocaine, procaine and tetracaine) and amino amide (bupivacaine, mepivacaine, lidocaine, prilocaine and dibucaine) local anesthetics (Fig. 1) and model membranes with emphasis on the contribution of differences in the structure and physicochemical properties of LA to

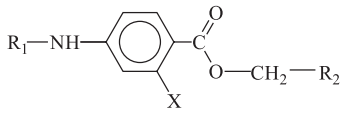
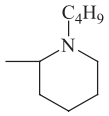
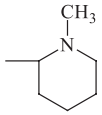
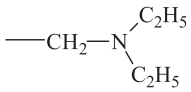
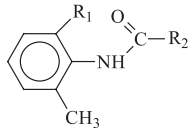
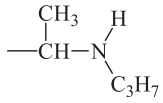
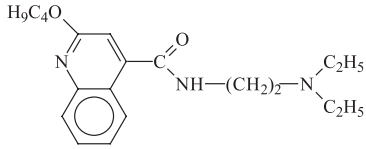
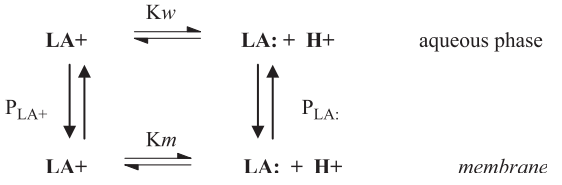
Anesthetic	Abbr.	R_1	R_2	X	Chemical Structure
Benzocaine	BZC	- H	- CH_3	-H	
Chloroprocaine	CLP	- H	- CH_2-N(C_2H_5)$_2$	- Cl	
Procaine	PRC	- H	- CH_2-N(C_2H_5)$_2$	- H	
Tetracaine	TTC	- C_4H_9	- CH_2-N(CH_3)$_2$	- H	
Bupivacaine	BVC	- CH_3			
Mepivacaine	MVC	- CH_3			
Lidocaine	LDC	- CH_3			
Prilocaine	PLC	- H			
Dibucaine	DBC				

Fig. 1. Chemical structure of the local anesthetics: ester (BZC), amino esters (CLP, PRC, TTC) and amino amides (BVC, MVC, LDC, PLC and DBC).

their effects on biological (erythrocyte) membranes. To characterize these effects, we determined neutral and charged LA partition into synthetic (phospholipid) bilayers and the biphasic hemolytic effect triggered by these compounds in erythrocytes at pH 7.4.

The study of LA interaction with model membranes is justified by the direct correlation between LA hydrophobicity and its potency and systemic mainly cardiovascular toxicity [1,3–5]. Charged and uncharged LA species seem to play a crucial role in anesthesia [5]. Most of the clinically used LA investigated in the present study are small amphiphilic molecules with a protonated amine group (pK_w around 8), except for BZC, an always uncharged ester type molecule. It is widely accepted that, once ionization equilibrium (K_w) is established in the extracellular aqueous phase, the uncharged form (LA:) rapidly crosses the lipid membrane bilayer to reach the cytosol, where the ionization equilibrium is reestablished [8] as follows:



Although both charged (protonated) and uncharged forms can coexist at physiological pH, it has been shown [8–10] that the K_w of anesthetics can be increased if measured in the presence of membranes, due to differential partition (P) of charged/uncharged forms into the membranes, leading to a higher ratio of uncharged species at pH 7.4.

The present study shows how important the contribution of the ΔpK triggered by the presence of membranes can be in the modulation of the biological effect of LA, or any other amphiphilic drugs that undergo ionization in the physiological pH range.

2. Materials and methods

Egg phosphatidylcholine (EPC), BVC, BZC, CLP, DBC, LDC, PRC and TTC were purchased from Sigma (St. Louis, MO), MVC was donated by Sterling Drug (Rensselaer, NY), and PLC by Cristália Produtos Químicos e Farmacêuticos (Itapira, SP). All other compounds were reagent grade. Except for BZC, all other anesthetics were obtained in the hydrochloride form.

2.1. Membrane preparation

EPC Multilamellar Vesicles were prepared by evaporating stock chloroform EPC solutions under a stream of wet nitrogen. The samples were left under vacuum for no less than 2 h. Vesicles were obtained by the addition of phosphate buffered saline (PBS) (0.15 M NaCl and 5 mM phosphate, pH 7.4) followed by vortexing for 5 min. *Erythrocytes*: freshly obtained mouse blood was collected in Alsever's solution (27 mM sodium citrate/72 mM NaCl/114 mM glucose/2.6 mM citric acid) and washed three times in PBS. The protocols were approved by the Institutional Animal Care and Use Committee of Unicamp, which follows the recommendations of the Canadian Council on Animal Care [11].

2.2. Partition coefficient (P) determination

Multilamellar vesicles were prepared as described above and kept in PBS. A known amount of LA was incubated with the membranes for 10 min at room temperature. The drug concentration remaining in the supernatant, after centrifugation at 105 000×g for 1 h, was optically detected at the appropriate wavelength for each LA, as described before [12,13]. The amount of drug bound to the lipid phase was obtained by subtracting the supernatant concentration from the total drug concentration, measured before phase mixing. The partition coefficient, P, was determined according to Eq. (1) [14]:

$$P = \frac{n_m/V_m}{n_w/V_w} \quad (1)$$

where: n is the number of moles of LA, V is the volume and the subscripts 'm' and 'w' refer to the membrane and aqueous phase, respectively. The apolar phase volume (V_m) was calculated assuming a lipid density of 1 g/ml [15–17]. Each P value represents the mean of four repetitions in three independent experiments (n=12).

2.3. Hemolytic assay under isotonic conditions

LA stock solutions were prepared in isotonic PBS solution and added to erythrocytes (0.14% hematocrit) prepared in the same buffer. The samples were kept at room temperature (22–25 °C) for 30 min before centrifugation at 260×*g* for 3 min. Hemoglobin released into the supernatant was detected at 412 nm.

The *hemolytic* effect, measured as percent hemolysis, was calculated from the released hemoglobin, as follows:

$$\text{Hemolysis } (\%) = \frac{A_s - A_{c1}}{A_{c2} - A_{c2}} \cdot 100 \qquad (2)$$

where A denotes the absorbance; 's' refers to the sample; 'c1' is the mechanical hemolysis control, i.e., the absorbance of erythrocytes in PBS, and 'c2' is the 100% hemolysis control, measured with the erythrocytes in water.

Whenever necessary (when high concentrations of LA were used), pH was corrected by the addition of small volumes of 0.1 M NaOH.

2.4. Hemolytic assay under hypotonic conditions

Erythrocytes (0.14% hematocrit) were incubated in a hypotonic buffer solution (5 mM phosphate, pH 7.4, plus 66 mM NaCl), which induced 50% hemolysis. Each LA, prepared in stock solutions of the same hypotonicity, was added to the erythrocytes and the samples were incubated for 30 min. After centrifugation at 260×*g* for 3 min, the released hemoglobin was measured in the supernatant at 412 nm. Results are expressed on a relative absorbance (R.A.) scale ranging from <1 (protection) to >1 (hemolysis), according to Eq. (3).

$$\text{R.A.} = \frac{A_s}{A_{c3}} \qquad (3)$$

where R.A.=1 indicates 50% hemolysis obtained in the presence of hypotonic buffer (c3). Each R.A. value represents the mean of six independent experiments.

3. Results and discussion

Table 1 shows some physicochemical properties of the anesthetics investigated here. Three different families were represented: esters, amino esters and amino amides. The ester (BZC) and amino esters (CLP, PRC, TTC) are benzoic acid derivatives. The amino amides bupivacaine, mepivacaine, lidocaine and prilocaine belong to a homologous series, with cyclic (BVC, MVC) or linear (LDC, PLC) substituents (Fig. 1). DBC is from a different amino amide series and presents a voluminous quinoline ring, which confers higher hydrophobicity on it compared to the other LA, together with steric hindrances that seem to play a crucial role in the association of DBC with model and biological membranes [18].

Table 1
Some physicochemical properties of the local anesthetics: molecular weight (MW), van der Waals area, water solubility (S_w) and EPC/water partition coefficient (P); LA+ and LA: refer to the charged and uncharged species, respectively

LA	MW	Area ($Å^2$)[a]	S_{wLA+} (M)[b]	$S_{wLA:}$ (mM)[c]	P_{LA+}[b]	$P_{LA:}$[c]	$P_{LA:}/P_{LA+}$
BZC	165.2	–	–	4.40	–	253±43	–
CLP	307.2	447	0.20	1.98	61±29	250±46	4.1
PRC	272.8	422	2.95	16.30	66±26	84±32	1.3
TTC	300.8	544	1.34	0.76	144±51	868±23	6.0
BVC	324.9	451	0.07	0.58	(96±6)	798±147	(8.3)
MVC	282.8	396	1.30	8.82	35±17	98±12	2.8
LDC	270.8	433	2.30	13.10	32±5.8	144±54	4.5
PLC	256.8	428	0.83	23.10	26±12	110±61	4.2
DBC	379.9	617	1.90	0.03	1790±545	2614±49	1.5

[a] According to Ref. [5].
[b] Determined in 0.2 M acetate buffer, pH 5.0 except for BVC (5 mM PBS, pH 7.1-values in brackets).
[c] Determined in 0.2 M carbonate buffer, pH 10.5.

Many of these properties have been discussed before, such as the differences in hydrophobicity (P and water solubility, S_w, values) and in the ionization constants (pK values) of the homologues, as well as their relation to the clinical properties of the LA [6,12,19]. Here we introduced, besides the data for BZC, the determination of P values for the charged LA species (P_{LA+}) so that we could compare the $P_{LA:}/P_{LA+}$ ratios for the LA studied. Although some of these compounds (MVC, BVC and PLC) are present as racemates, we found no significant changes in S_w and P values between the enantiomers (data not shown).

The partition of amphipathic compounds between phospholipid vesicles and water is of the same order of magnitude as that observed between erythrocytes and other biological membranes/water [13,20,21]. EPC liposomes were used as a model for the eryth-

rocyte membrane partition because they do not have proteins to be denatured by pH extremes and also because they allow pH measurements ranging from pH 4 to 12, without changing the ionization state of the phospholipid molecules [10,22].

Table 1 shows that PRC and DBC have the smallest uncharged/charged ratios of partition ($P_{LA:}/P_{LA+}$) and that BZC is always present in a non-ionizable form. The quinoline ring of dibucaine and its *o*-butyl substituent guarantees a high degree of hydrophobicity to the molecule, even in the charged species, in comparison to the other LA studied. This equivalency between $P_{LA:}/P_{LA+}$ values for DBC agrees well with data reported by Eftink et al. [23] who described values of P=2570 and 1260 for the partition of uncharged and charged forms of DBC, respectively, between dimyristoyl phosphatidylcholine liposomes and water. PRC, on the other hand, is the least hydrophobic of the LA studied and its benzoate structure confers a small difference in the P values of PRC:/PRC+.

For experimental reasons, we were unable to determine the partition coefficient of BVC+. Its small molar absorption coefficient in the UV [12] did not allow the quantification of the small amounts of drug remaining in the supernatant at pH 5.0. This finding is surprising since we had no problems in P_{LA+} determination with MVC, the other cyclic amino amide LA, but with a methyl instead of a butyl substitution in the piperidine ring. Since the van der Waals areas of these molecules are quite different—451 and 396 $Å^2$ for BVC and MVC, respectively [5]—we wonder if steric hindrances could be limiting the insertion of charged BVC species into the membrane, as described for DBC [18]. We determined the partition coefficient of BVC at pH 7.1 and the small value obtained (P=96) indicated a great difference between the P values ($P_{LA:}/P_{LA+}$ ratio >8—Table 1), comparable to that of the amino ester TTC, with similar hydrophobicity.

3.1. Ratio of uncharged/charged LA species at pH 7.4

An overall view of the results presented in Table 1 shows that LA have quite different P values for their charged/uncharged forms, and that the neutral form binds strongly ($P_{LA:}>P_{LA+}$) to the membrane than the protonated, less hydrophobic species.

Based on the Gouy-Chapman theory, Lee [9] analyzed the binding of ionizable compounds (amine LA) to lipid bilayers and showed that whenever the binding of charged/uncharged forms is different there will be a pK shift of the partitioning compound. Electron spin resonance studies by Schreier et al. [8] demonstrated that the ionization constant of tetracaine down-shifts from 8.5 to 7.0 in the presence of egg PC multilamellar vesicles. The effect of different partitioning on the ionization constant of a large number of ionizable compounds in micellar surfactant systems [24] and of local anesthetics on lipid membranes has been reviewed elsewhere [6].

In Table 2 we applied the formalism described by Lee and Schreier [10] to calculate pK_{app}, the apparent ionization constant, measured in water but in the presence of membranes:

$$pK_{app} = pK - \log[(P_{:} \cdot V_m + V_w)/(P_{+} \cdot V_m + V_w)] \quad (4)$$

Simulating the physiologic condition of 50% hematocrit (erythrocytes in blood) in which the concentration of lipids in the membrane is 2.8 mg/dl [20], the

Table 2
Ionization constants for the local anesthetics (LA) measured in water (pK_w) and calculated in the presence of the membrane (pK_{app})

LA	pK_w	pK_{app}	ΔpK	R_w [a,b]	R_{app} [a,c]	$P_{average}$ [d]
BZC[e]	–	–		∝	∝	253[d]
CLP	9.0[f]	8.85	0.15	0.025	0.035	67
PRC	9.0[f]	8.98	0.02	0.025	0.033	67
TTC	8.5[f]	8.11	0.39	0.079	0.200	236
BVC[e]	8.1[f,g]	–	–	0.199	–	–
MVC	7.6[f]	7.54	0.06	0.630	0.730	62
LDC	7.8[f,h]	7.69	0.11	0.398	0.650	76
PLC	7.8[f]	7.61	0.19	0.398	0.620	58
DBC	8.3[h]	8.13	0.17	0.125	0.180	1920

Uncharged/charged ratios of LA at pH 7.4, considering pK_w (R_w) and pK_{app} (R_{app}), and average partition coefficient at pH 7.4 ($P_{average}$) considering R_{app}. Calculations based on the EPC/water partition coefficients for the LA at 22 °C (Table 1).

[a] Molar ratio of uncharged/charged LA species at pH 7.4, calculated by the Henderson–Hasselbalch equation.

[b] Using pK_w values.

[c] Using pK_{app} values.

[d] Partition coefficients at physiological pH, calculated from the real uncharged/charged molar ratio at pH 7.4 (R_{app}) in Eq. (6).

[e] Calculations are not given for BZC and BVC since their P values for the charged species could not be determined.

[f] According to Ref. [40].

[g] According to Ref. [41].

[h] According to Ref. [2].

apolar phase volume (V_m) was calculated and pK_{app} was obtained according to Eq. (4). The data in Table 2 show that in the presence of membranes, a down-shift in the ionization constants of the LA can be found, varying in the 0.02–0.39 pH unit range for this membrane concentration.

Using pK_{app} in the Henderson–Hasselbalch equation (Eq. (5)), one can see that the neutral (LA:) to charged (LA+) molar ratio at pH 7.4 increases considerably in the presence of membranes (R_{app}—Table 2) in comparison to the ratio in water (R_w). R_{app} values were lower than unit for all of the anesthetics studied; in spite of the LA+ predominance at physiologic pH, the presence of the neutral LA species at pH 7.4 was greater than expected. In the case of lidocaine and prilocaine, R_{app} get closer to one and for TTC (and probably BVC) it was more than twofold higher than R_w.

$$\mathrm{pH} = \mathrm{p}K + \log[\mathrm{base}]/[\mathrm{acid}] \quad (5)$$

The average partition coefficient at pH 7.4 ($P_{average}$) was calculated taking into account these LA:/LA+ ratios in the presence of membranes (R_{app}, Table 2) and the measured values of $P_{LA:}$ and P_{LA+}, according to Eq. (6):

$$P_{average} = \frac{P_{LA+} + (P_{LA:} \cdot R_{app})}{1 + R_{app}} \quad (6)$$

The increment in the uncharged/charged species ratios that occurs in the presence of membranes at pH 7.4 triggers an increase in $P_{average}$ values that would not be predicted in water. In fact, the P value for pH 7.4 taken from R_w (pK_w values) would be lower than $P_{average}$ (corrected by the real uncharged/charged ratios in the presence of membranes). For instance, in the case of TTC, at pH 7.4, P would be 159 instead of 236 ($P_{average}$, Table 2), i.e., 1.5 times lower than it really is in the presence of (2.8 mg/dL lipids) membranes.

For BVC the difference between $P_{LA:}$ and P measured at pH 7.1 (Table 1) was of one order of magnitude, so that, ΔpK must be as high as that of TTC and $P_{average}$ will receive a strong contribution from the uncharged LA species. Taken together, these calculations lead us to conclude that P_{BVC+} is really very low, as discussed earlier.

The data in Table 2 clearly show that to evaluate the effect of ionizable compounds in which both species are present at physiological pH, the partition of each species should be considered, since it can change the percentage of uncharged/charged forms present under the experimental conditions used.

3.2. Hemolytic studies

Since the pioneering work of Seeman [25] many papers in the literature have demonstrated that low LA concentrations protect erythrocytes from hyposmotic hemolysis, while high concentrations induce hemolysis both in hyposmotic and isosmotic conditions [13,25–28]. Fig. 2 exemplifies this biphasic effect of anesthetics, using DBC: (a) the protective action

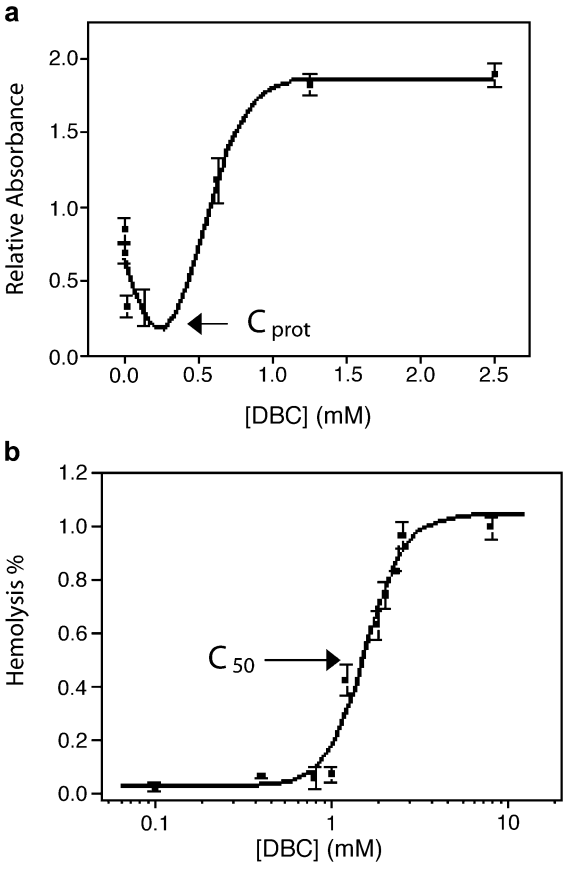


Fig. 2. Hemolytic effect of dibucaine under hypotonic (a) and isotonic (b) conditions. 0.14% Ht, pH 7.4, 30 min incubation at room temperature.

against hemolysis of small amounts of DBC in erythrocytes exposed to a hypotonic medium, and b) the lysis, seen as a sigmoid curve, when erythrocytes were exposed to the higher LA concentration in isosmotic condition. From plots like those in Fig. 2, we determined two hemolytic parameters: C_{prot}, the LA concentration for maximum protective effect (Fig. 2a) [20], and C_{50}, the concentration needed to induce 50% hemolysis in isotonic medium (Fig. 2b).

Table 3 reports potency as well as C_{prot} and C_{50} values determined for all the LA studied. Both C_{prot} and C_{50} are strongly related to the partition of LA into membranes, since the more hydrophobic anesthetics DBC, TTC and BVC have the highest hemolytic effects (low C_{prot} and C_{50} values). The opposite can also be observed, since the more hydrosoluble anesthetics PLC, MVC, LDC, CLP and PRC with low $P_{average}$ values (Table 2) showed high C_{prot} and C_{50} values (Table 3). BZC did not show any hemolytic effect up to 4.4 mM; in a recent paper we showed that this LA molecule has a limited effect on membranes due to its low water solubility and non-ideal partition [13]. In the cited study we demonstrated that if the LA was prepared in buffered saline solution containing 5 mol% DMSO, the hemolytic effect of BZC could be seen, leading to C_{prot} and C_{50} values of 13 and 15.5 mM, respectively [13] at the hematocrit used here. In the presence of DMSO the partition coefficient of benzocaine decreases to 115 [13] so that the hemolytic effect of BZC fits well the data in Table 3.

The protective effect: DBC>BVC≥TTC>MVC>LDC>PLC>CLP>PRC reflects well the profile of $P_{average}$ values presented here. The correlation coefficient for logC_{prot} vs. log$P_{average}$ (0.835, n=8) was quite similar to that of logC_{prot} vs. logPotency (r=0.865, n=8). C_{50} values showed poorer results (logC_{50} vs. log$P_{average}$ r=0.797 and logC_{50} vs. logPotency r=0.867, n=8). The better correlation obtained with C_{prot} vs. $P_{average}$ was expected since the protective effect is mainly dependent on the insertion of the amphiphile molecule into the bilayer [29] while membrane solubilization (hemolysis) depends also on phenomena like amphiphile–amphiphile interactions inside the membrane and in the aqueous phase [30,31].

Table 3
Potency and hemolytic parameters of the local anesthetics: C_{prot} and C_{50}, the concentration inducing a maximum protective effect and 50% hemolysis, respectively

LA	Potency[a]	C_{prot} (mM)	C_{50} (mM)
BZC	1.2	–	–
CLP	4	80.0	87
PRC	1	133	300
TTC	16	1.2	5.5
BVC	16	1.0	8.0
MVC	2	9.9	27.7
LDC	4	16.1	>140
PLC	3	50	60.8
DBC	16	0.12	1.8

Ht 0.14%, pH 7.4, 30 min incubation at 22 °C.
[a] According to Ref. [1].

Although many studies dealing with the effect of LA on model membranes are available in the literature (see Ref. [6] for a review), in an interesting recent article [7] comparing the effect of TTC, PRC, BVC, LDC and PLC on three different preparations of synaptosomal membranes, the authors found a direct correlation between the strength profile of the LA effect on these model membranes—measured as perturbation of the hydrocarbon core—and the P values of the anesthetics, at pH 7.4.

As discussed before, $P_{average}$ values indicate that partitioning is higher than one would expect by ignoring the pK shift induced by the presence of membranes. Besides the fact that the predominant form of LA in the hemolytic experiments (pH 7.4) is the protonated (or charged) one, it seems that the uncharged form plays also a significant role in the hemolytic effect. This explains the direct correlation between the hydrophobicity and biological effects of LA [1,6,7,25,26,32–34].

4. Conclusion

When studying the mechanism of action of ionizable pharmacological compounds on the membrane, one should take into account that the ionized and non-ionized forms will probably have different partitions between membrane/water. As shown here, this fact should not be neglected in the quantification of the effective amount of drug for the membrane effect. The site of action of anesthetics is the voltage-gated sodium channel, an integral membrane protein; in myelinized nervous fibers the amount of bilayers guarantees that almost 100% of the clinically used

LA studied here will reside inside the lipid bilayer. At physiological pH and at this high membrane concentration, the pH at which the LA is half-ionized downshifts considerably (Table 2). This phenomenon is even more relevant if one of the forms of the ionizable compound is more active. We should remember that early in the 1970s, Narahashi et al. [35,36], using quaternary amine LA and isolated nervous fibers, suggested that the active form of local anesthetics was the protonated one, when it reached the voltage-dependent sodium channel from the inner side of the axon. Conversely, the development of molecular biology has allowed the determination of the sequence of the sodium channel protein [37] and, in more recent years, site-directed mutagenesis has provided strong evidence for the existence of a hydrophobic site in the channel, inside the bilayer, for the binding of uncharged LA [38,39].

In conclusion, the good correlation between the hemolytic activity, P_{average} and Potency of the LA investigated in the present study reinforces the importance of P_{average} determination and reveals that the hemolytic assays can be used as an alternative tool to predict the pharmacological properties of amphiphilic compounds on membranes.

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